Tryptase/Protease-Activated Receptor 2 Interactions Induce Selective Mitogen-Activated Protein Kinase Signaling and Collagen Synthesis by Cardiac Fibroblasts

Jennifer L. McLarty, Giselle C. Meléndez, Gregory L. Brower, Joseph S. Janicki, Scott P. Levick

Abstract—The mast cell product, tryptase, has recently been implicated to mediate fibrosis in the hypertensive heart. Tryptase has been shown to mediate noncardiac fibroblast function via activation of protease-activated receptor 2 and subsequent activation of the mitogen-activated protein kinase pathway, including extracellular signal–regulated kinase 1/2. Therefore, we hypothesized that this pathway may be a mechanism leading to fibrosis in the hypertensive heart. Isolated adult cardiac fibroblasts were treated with tryptase, which induced activation of extracellular signal–regulated kinase 1/2 via protease-activated receptor 2. Blockade of protease activated receptor 2 with FSL LRY (10 μmol/L) and inhibition of the extracellular signal–regulated kinase pathway with PD98059 (10 μmol/L) prevented collagen synthesis in isolated cardiac fibroblasts stimulated with tryptase. In contrast, p38 mitogen-activated protein kinase and stress-activated protein/c-Jun N-terminal kinase were not activated by tryptase. Cardiac fibroblasts isolated from spontaneously hypertensive rats showed this same pattern of activation. Treatment of spontaneously hypertensive rats with FSL LRY prevented fibrosis in these animals, indicating the in vivo applicability of the cultured fibroblast findings. Also, tryptase induced a myofibroblastic phenotype indicated by elevations in α-smooth muscle actin and extra type III collagen levels in spontaneously hypertensive rats. We found that tryptase acts via PAR-2 to induce ERK1/2 but not p38 or SAPK/JNK activation in isolated adult rat cardiac fibroblasts. However, the mechanism by which tryptase is able to elicit this response is unknown. Tryptase and trypsin are the major activators of protease-activated receptor 2, but do not activate PAR-1, -3, and -4.

We recently presented evidence identifying the cardiac mast cell as central to the induction of fibrosis in the hypertensive rat heart. The mast cell product, tryptase, is associated with the pathogenesis of fibrosis in many tissues, particularly that of the lung, kidney, and skin. We further identified that tryptase is elevated in the hypertensive heart and that tryptase stimulates collagen production and proliferation by isolated adult cardiac fibroblasts. However, the mechanism by which tryptase is able to elicit this response is unknown. Tryptase and trypsin are the major activators of protease-activated receptor (PAR) 2, but do not activate PAR-1, -3, and -4. The PARs represent a novel group of G protein–coupled 7 transmembrane-domain receptors that are activated by serine proteases. These proteases cleave the N-terminal receptor sequence at SKGR↓ SLIGRL sites, exposing the sequence of the tethered ligand (SLIGRL for rat PAR-2). This tethered ligand binds to a site on the second extracellular loop of the receptor, triggering its autoactivation and subsequent signaling cascade events, such as mitogen-activated protein kinase (MAPK) phosphorelay, which includes extracellular signal–regulated kinase isoforms 1 and 2 (ERK1/2), p38, and stress-activated protein kinase/c-Jun N-terminal kinases (SAPK/JNK). There are only a few descriptions of tryptase/PAR-2 signaling events in any type of fibroblast, with Frugieri et al having reported that tryptase/PAR-2 signaling involves the activation of ERK1/2 in fetal foreskin fibroblasts.

Accordingly, the present study sought to determine whether tryptase activates cardiac fibroblasts via PAR-2 and to identify whether specific MAPK pathways are involved. We found that tryptase acts via PAR-2 to induce ERK1/2 but not p38 or SAPK/JNK activation in isolated adult rat cardiac fibroblasts. Activation of this cascade led fibroblast to transition to a myofibroblastic phenotype and increased secretion of fibronectin.
of collagen. This pathway was also found to be active in cardiac fibroblasts isolated from hypertensive rat hearts. This study is the first to establish PAR-2 activation in cardiac fibroblasts and the selective downstream activation of ERK1/2.

Methods

Rats were housed under standard environmental conditions, maintained on commercial rat chow and tap water ad libitum. All of the studies conformed to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of South Carolina Institutional Animal Care and Use Committee. Anesthesia at the experimental end point was affected by sodium pentobarbital (50 mg/kg, IP).

Cardiac Fibroblast Isolation and Culture Studies

Cardiac fibroblasts were obtained from adult male Sprague Dawley rats (8 weeks old, n=4), spontaneously hypertensive rats (SHR; 12 weeks old, n=4), and Wistar Kyoto rats (WKY; 12 weeks old, n=4). Briefly, the left ventricle (LV) was minced and digested with Liberase 3 (Roche) and fibroblasts purified by selective attachment to plastic culture ware. Fibroblasts were maintained in DMEM containing 10% neonatal bovine serum and 5% fetal calf serum with replacement of media every other day. All of the fibroblasts were used on the second passage to minimize changes in phenotype associated with culture. Before experimentation, 1 million fibroblasts were seeded on 100-mm plastic culture plates and allowed to adhere to the plate for 24 hours in DMEM with 10% neonatal bovine serum and 5% fetal calf serum. Next, cells were rinsed with MosconaSalt Solution and the media replaced with serum-free DMEM-F12 media for 24 hours. For the studies assessing collagen production, fibroblasts were either untreated or treated with tryptase (1000 mU, Promega) dissolved in DMEM with 1.5% FBS for 24 or 72 hours. In addition, tryptase-treated fibroblasts were pretreated for 1 hour with the PAR-2 peptide antagonist (FSLLRY; Peptides International) or the MAPK kinase (MEK) 1/2 inhibitor (PD98059, 10 μmol/L, Cayman Chemical). PD98059 was used because MEK1/2 is immediately upstream of ERK1/2, thus, inhibiting its effects. For MAPK activation experiments, fibroblasts were incubated with tryptase, tryptase in the presence of FSLLRY or PD98059 for 10 minutes. At the completion of the experiments, fibroblasts and media were collected and snap frozen.

Collagen Production by Isolated Cardiac Fibroblasts

Adult cardiac fibroblasts production of collagen was determined by hydroxyproline analysis of media, as described by Edwards and O’Brien* (please see the online Data Supplement at http://hyper.ahajournals.org).

Western Blot Analysis

Please see the online Data Supplement.

MAPK Activation

Please see the online Data Supplement.

Whole Animal Studies

Twelve-week-old SHR were randomly assigned to 2 groups, untreated (n=6) and those treated with the PAR-2 peptide antagonist FSLLRY (10 μg/kg per day, IP, Peptides International; n=5), with the experimental end point being at 15 weeks of age. At 12 weeks of age, SHR are known to be hypertensive.9 This dosage of FSLLRY was based on previous work by Al-Ani et al.10 Untreated WKY rats served as age-matched controls. At the experimental end point, measurements of systolic, diastolic, and mean arterial pressures were made using a catheter attached to a pressure transducer and inserted into the right carotid artery. After euthanization, the heart was removed and the atria and great vessels dissected away, with the LV and right ventricle (RV) separated and weighed. A transverse midsection of the LV was placed in 4% paraformaldehyde for fixation. Lung weight was also obtained after the removal of the esophagus and trachea with the pleural surface blotted dry.

Analysis of Left Ventricular Collagen Volume Fraction

Paraffin-embedded blocks were prepared from midventricular sections of the LV as described previously.1 Five-micrometer thick sections were stained with collagen specific picrosirius red (0.1% Sirius Red F3BA in picric acid). Photomicrographs of 20 random fields, avoiding perivascular collagen, were obtained using a Ziess Axiosvert 200 fluorescent microscope with 20× objective. Collagen volume fraction was determined from these images using Image J and expressed as a percentage of the myocardial area.11

Statistical Analysis

Data are expressed as mean±SD or SEM as appropriate. The results were analyzed statistically by using the computer program SPSS (Chicago, IL). Quantitative data were compared among the multiple groups by one-way ANOVA followed by Bonferroni post hoc
testing. For 2 group comparisons, the Student t test was used. Differences were considered to be significant when \( P < 0.05 \).

**Results**

**Tryptase/PAR-2 Induces Selective MAPK Activation in Isolated Cardiac Fibroblasts**

Knowing that tryptase induces collagen synthesis by isolated cardiac fibroblasts,\(^1\) we sought to determine which specific MAPK pathways were activated by tryptase. There was a significant increase in the level of total ERK in isolated cardiac fibroblasts treated with tryptase. Nevertheless, the ratio of phosphorylated ERK to total ERK significantly increased by 143% compared with control after stimulation with tryptase (\( P < 0.01 \) versus control; Figure 1A). This ERK activation was induced by tryptase activation of PAR-2, because the PAR-2 antagonist FSLLRY prevented the increase in the ratio of phosphorylated ERK to total ERK (\( P < 0.01 \) versus tryptase). Pretreatment with MEK1/2 inhibitor PD98059 reduced phosphorylated ERK to values below the detectable limits of the ELISA, indicative of its effectiveness (\( \leq 38.3 \) pg/mL; data not shown). For p38 and SAPK/JNK, there were no significant differences in the ratio of phosphorylated to total protein between the tryptase-treated group and the controls (Figure 1B and 1C).

**Isolated Cardiac Fibroblast PAR-2 Levels**

The presence of PAR-2 was examined on isolated adult cardiac fibroblasts. PAR-2 was detected by Western blot on untreated fibroblasts (Figure 2). PAR-2 levels increased above that of untreated cells after 72 hours but not 24 hours of tryptase treatment. This was prevented by blockade of PAR-2.

**Collagen Production by Tryptase Treated Isolated Cardiac Fibroblasts**

Isolated adult cardiac fibroblasts were treated with tryptase, tryptase together with the PAR-2 antagonist FSLLRY, or tryptase with the MEK1/2 inhibitor PD98059 for 24 and 72 hours and hydroxyproline concentration in the media measured as a marker for collagen (Figure 3). Tryptase significantly increased collagen production at both 24- and 72-hour time points. PAR-2 blockade prevented collagen production at both time points. The MEK1/2 inhibitor PD98059 also prevented collagen production at both 24 and 72 hours.

**Figure 2.** Representative image (A) of a Western blot detecting protease-activated receptor (PAR) 2 on isolated adult cardiac fibroblasts. Ratio of PAR-2 to GAPDH on isolated cardiac fibroblasts after stimulation with tryptase for (B) 24 hours and (C) 72 hours. *\( P < 0.05 \) vs control.

**Figure 3.** Hydroxyproline concentration in media from isolated adult cardiac fibroblasts treated for 24 hours (A) and 72 hours (B). Adult cardiac fibroblasts were treated with tryptase, tryptase + FSLLRY (protease-activated receptor [PAR] 2 antagonist), or tryptase + PD (PD98059; mitogen-activated protein kinase kinase [MEK] 1/2 inhibitor). All of the values are mean±SEM; *\( P < 0.05 \) vs control; †\( P < 0.05 \) vs tryptase.
Isolated Cardiac Fibroblast α-Smooth Muscle Actin and ED-A Fibronectin Levels

Tryptase treatment induced a trend toward increased α-smooth muscle actin compared with control, but this increase did not reach significance ($P=0.067$; Figure 4A and 4B). Pretreatment with FSLLRY or PD98059 prevented this trend. Tryptase treatment significantly increased ED-A fibronectin levels in adult cardiac fibroblasts ($P<0.001$ versus control). Pretreatment with FSLLRY or PD98059 prevented the induction of the myofibroblast marker, ED-A fibronectin, in adult cardiac fibroblasts ($P<0.001$ for both groups versus tryptase, Figure 4C and 4D).

MAPK Activation in SHR Fibroblasts

To determine whether the pattern of MAPK pathway activation was the same in fibroblasts from hypertensive hearts, fibroblasts were isolated from SHR and the three MAPK pathways examined. SHR fibroblasts had significant increases in the ratio of phosphorylated to total ERK compared with WKY fibroblasts (0.218±0.055 and 0.027±0.004, $P=0.0013$, respectively; Figure 5A). There were no differences in the ratio of phosphorylated to total protein of p38 and SAPK/JNK in the SHR compared with the WKY fibroblast (Figure 5B and 5C).

Whole Animals Studies

Having identified PAR-2 as a mechanism by which tryptase activates cardiac fibroblasts, we then sought to determine whether this receptor did in fact play a role in fibrosis in vivo in the hypertensive heart. Table contains the average values for the biometric measurements for WKY, SHR, and SHR+FSLLRY animals. Although the LV weights and the LV to body weight ratio of the SHR and SHR+FSLLRY animals

Figure 4. Representative Western blot (A) for α-smooth muscle actin in isolated adult cardiac fibroblasts. Adult cardiac fibroblasts were treated with tryptase, tryptase+FSLLRY (protease-activated receptor [PAR] 2 antagonist), or tryptase+PD (PD98059; mitogen-activated protein kinase [MEK] 1/2 inhibitor) for 24 hours. Graphic representation (B) of α-smooth muscle actin in adult cardiac fibroblasts treated with tryptase, tryptase+FSLLRY, or tryptase+PD. All of the values are mean±SEM, *$P<0.05$ vs tryptase. Representative Western blot (C) for extra type III domain A (ED-A) fibronectin in isolated adult cardiac fibroblasts treated as previously stated. Graphic representation (D) of ED-A fibronectin in isolated adult cardiac fibroblast treated as stated previously. All of the values are mean±SEM, *$P<0.05$ vs control; †$P<0.05$ vs tryptase.

Figure 5. Ratio of total to phosphorylated (A) extracellular signal–regulated kinase isoforms 1 and 2 (ERK1/2), (B) p38, and (C) stress-activated protein kinase/c-Jun N-terminal kinases (SAPK/JNK) in cardiac fibroblasts isolated from spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats. All of the values are mean±SEM, *$P<0.05$ vs WKY.
were significantly increased from that of the WKY animals, these 2 groups were not significantly different from each other. Right ventricle weights were not significantly different between any of the groups. SHR and SHR/H11001FSLLRY had significant increases in systolic blood pressure, diastolic blood pressure, and mean arterial pressure compared with the WKY animals but were not significantly different from each other. Collagen volume fraction was significantly increased in the SHR group compared with the WKY group (Figure 6). Collagen volume fraction was normalized in the SHR/H11001FSLLRY group (P<0.05 versus SHR).

Discussion

We recently found that cardiac mast cells can induce fibrosis in the hypertensive heart and that tryptase levels were increased in these hearts.1 We further demonstrated that tryptase was able to induce proliferation and collagen production by isolated adult cardiac fibroblasts. The stimulatory action of tryptase on fibroblast function is thought to be a PAR-2–mediated event;5 however, this had not been investigated in cardiac fibroblasts. The major finding of this study is that tryptase induces collagen production in cardiac fibroblasts via the activation of PAR-2 and subsequent selective induction of MAPKs.

PAR-1, -2, -3, and -4 are found in a variety of cells, including many cell types throughout the cardiovascular system. PAR-1 has been shown to be expressed by cardiac fibroblasts and able to induce proliferation through the activation of ERK1/2.12 However, whereas tryptase activates PAR-2, it is not a ligand for PAR-1, -3, and -4. To date, little is known about PAR-3 and PAR-4 activation in cardiac fibroblasts. This is the first study to examine PAR-2 activation in the cardiac fibroblast.

PAR-2 has been described in cardiac tissue; in fact, the expression of PAR-2 is present as early as embryonic day 17 in mice and is constitutively expressed in the adult murine heart.13 Bohm et al14 also reported PAR-2 transcript expression to be present in the adult human heart. Although the location of this receptor was previously thought to be mainly on cardiomyocytes, we established herein that isolated adult cardiac fibroblasts also have PAR-2. We show that PAR-2 levels in isolated adult cardiac fibroblasts increase in the presence of tryptase; however, this increase may be because of proliferation of the cardiac fibroblasts caused by tryptase.1

Frungieri et al6 reported that tryptase/PAR-2 activation, leading to phosphorylation of the MAPK ERK1/2, increased the fibrotic response in skin fibroblasts. However, it was unknown whether tryptase activation of PAR-2 activates this

Table. Biometric Data

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All of the values are mean±SD. BW indicates body weight; LV, left ventricle; RV, right ventricle; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; WKY, Wistar Kyoto rat; SHR, spontaneously hypertensive rat.

*P<0.05 vs WKY.

Figure 6. A, Representative images of picrosirius red-stained left ventricle (LV) sections from Wistar Kyoto (WKY) rats, spontaneously hypertensive rats (SHR), and SHR/H11001FSLLRY. B, Graphical representation of LV collagen volume fraction. All of the values are mean±SEM, *P<0.05 vs WKY; †P<0.05 vs SHR.
same pathway in cardiac fibroblasts. The potential role of MAPKs in mediating tryptase-induced fibrosis in the heart had also been unexplored previously. MAPK signaling includes the activation of the serine/threonine kinases, ERK1/2, p38 isoforms, and SAPK/JNK, all of which have extensive homology to one another. Growth and differentiation factors that induce early activation of ERK do so by sequential phosphorylation of the protein kinases Raf-1, MEK 1 and 2, and then ERK1/2 in most cells.15 We now show for the first time that there is an increase in ERK1/2 phosphorylation in isolated adult cardiac fibroblasts after treatment with tryptase. PAR-2 antagonism prevented ERK 1/2 phosphorylation and also prevented collagen production by cardiac fibroblasts. Furthermore, the MEK1/2 inhibitor PD98059 prevented collagen synthesis by cardiac fibroblasts stimulated with tryptase. This suggests that tryptase binding to the PAR-2 receptor causes signaling events through ERK1/2 to elicit the fibrotic response, whereas p38 and SAPK/JNK do not appear to be involved (Figure 7).

ERK1/2 has been shown to regulate procollagen gene expression in cardiac fibroblasts. ERK1/2 translocates to the nucleus to act directly on the α(I) procollagen gene. The transcription factors Egr, Ets, and AP-1 families are downstream of ERK1/2.16–18 Specifically, the α(I) procollagen gene contains both AP-1 and Ets binding sites in the 5’ untranslated region and first intron, and, therefore, these sequences may represent downstream targets of ERK1/2.19–21 Consequently, α(I) collagen has been shown to be a biomarker for fibrosis in the hypertensive hearts of humans and rats.22–24 To determine whether the same pattern of MAPK activation occurred in cardiac fibroblasts in the hypertensive heart, we isolated these cells from SHR and compared MAPK activation to that in fibroblasts from normotensive WKY hearts. We found that SHR cardiac fibroblasts had increased phosphorylation of ERK1/2 with no upregulation of p38 and SAPK/JNK, similar to what we observed in the isolated cultured fibroblasts treated with tryptase. This is consistent with our previous findings that tryptase is increased in SHR hearts.1

Although together our data show that tryptase stimulation of PAR-2 on isolated cardiac fibroblasts induces collagen synthesis, we wanted to know whether PAR-2 was in fact important in hypertensive hearts in vivo. Therefore, we treated SHR with the PAR-2 antagonist peptide FSLLRY and found that fibrosis was prevented. However, this treatment did not prevent LV hypertrophy or hypertension itself. This result is identical to what we have shown previously with the mast cell stabilizer, nedocromil,1 strengthening the concept that mast cell tryptase is a major driver of fibrosis in the hypertensive heart. This increase in collagen is a result of increased synthesis of both collagen types I and III by myofibroblasts and an imbalance of collagens metabolism by matrix metalloproteinases in the SHR.25 Myofibroblast’s ability to modulate collagen turnover contributes to tissue remodeling in the hypertensive heart, which ultimately leads to fibrosis.25 The phenotypic change of fibroblast to myofibroblast can be detected by the presence α-smooth muscle actin and ED-A fibronectin in the fibroblast.26,27 We found a trend toward increased α-smooth muscle actin and a significant increase in ED-A fibronectin in isolated cardiac fibroblasts treated with tryptase. These markers are indicative of fibroblasts transitioning to a myofibroblastic phenotype and suggest that tryptase and MAPK pathways can cause phenotypic conversion of the cardiac fibroblast to myofibroblast, which was prevented by blockade of PAR-2 and MAPK signaling pathway.

In summary, the results of this study reveal a previously unidentified pathway of cardiac fibroblast activation. This pathway is initiated by tryptase stimulation of PAR-2 and leads to induction of MAPK activation in the form of phosphorylation of ERK1/2. The phosphorylation of ERK leads to fibroblast conversion to a myofibroblastic phenotype and ultimately collagen production and accumulation within the heart. It is concluded that mast cell tryptase plays a critical role in the fibrosis of the hypertensive heart.

Perspectives

Because tryptase is derived almost exclusively from mast cells, the results of this study reinforce the importance of mast cells in the development of fibrosis in the hypertensive heart, which further extends our knowledge of how tryptase induces fibrosis in the heart. Our data now establish tryptase activation of PAR-2 in cardiac fibroblasts and the subsequent selective activation of ERK1/2 as being involved in the transition of fibroblast to myofibroblastic phenotype and increasing collagen synthesis. Thus, this study represents another indicator of the importance of mast cells to myocardial remodeling by describing a previously unknown role for tryptase/PAR-2. In addition, this study also highlights the importance of developing novel therapeutics that can target this receptor.

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Disclosures

None.
References


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TRYPTASE/PAR-2 INTERACTIONS INDUCE SELECTIVE MAPK SIGNALING AND COLLAGEN SYNTHESIS BY CARDIAC FIBROBLASTS.

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Collagen Production by Isolated Cardiac Fibroblasts.
Briefly, 6N HCL was added to all media samples in a 1:1 ratio and samples were maintained at 108°C overnight, filtered, and subjected to vacuum centrifugation. The samples were reconstituted in citrate buffer (0.2 M) and reacted with chloramine-T for 20 min, followed by incubation with aldehyde for 20 minutes, before cooling on ice. The absorbance was read at 550 nm and compared to hydroxyproline standards ranging from 0 to 10 μg/mL.

Western Blot Analysis.
Total protein was extracted from isolated adult rat cardiac fibroblasts by homogenization of the cells with buffer containing protease inhibitor cocktail (Pierce). Protein concentrations were determined by Bio-Rad Protein Assay. 35 μg of total protein was then loaded and separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane (BioRad). Ponceau-S staining was used to confirm equal loading and accurate transfer of proteins from the SDS-PAGE gel to the nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBS-0.01 % Tween for 2 hours at room temperature. For detection of PAR-2, the blots were probed with mouse anti-PAR-2 antibody (1:500, Santa Cruz Biotechnology) for 2 hours at room temperature, and then goat anti-mouse secondary antibody (1:1000, Santa Cruz Biotechnology) for 2 hours at room temperature. For α-smooth muscle actin (α-SMA), the blots were incubated with mouse anti-α-SMA antibody (1:2000, Santa Cruz Biotechnology) for 2 hours at room temperature. For ED-A fibronectin, the blots were incubated with mouse anti-ED-A fibronectin antibody (1:1000, Abcam) overnight at 4°C. The blots were subsequently incubated in goat anti-mouse secondary antibody as stated previously. Blots were also probed for GAPDH, which served as a loading control [(Primary Antibody: mouse anti-GAPDH, 1:3000 at room temperature for 2 hours, Santa Cruz Biotechnology); (Secondary Antibody: goat anti-mouse IgG2a, 1:5000 at room temperature for 2 hours, Santa Cruz Biotechnology)]. Immunoreaction signals were visualized with enhanced chemiluminescence (Pierce) by exposure to hyperfilm (Phenix Research Products). Densitometry analysis was performed with the BioRad GS-800 Calibrated Densitometer. PAR-2, αSMA, and ED-A fibronectin were quantified as a ratio to GAPDH.

MAPK Activation.
Total ERK and phosphorylated ERK were determined using commercially available ELISA kits from Assay Designs (Ann Arbor, MI). Total p38 and phosphorylated p38 were determined by commercially available ELISA kits from Enzo Life Science (Plymouth Meeting, PA). Total SAPK/JNK and phosphorylated SAPK/JNK were determined by commercially available ELISA Kits from Cell Signaling Technology (Beverly, MA). All determinations were performed using manufactures’ protocols.